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(54) Title: RESISTANCE TO ACETOHYDROXYACID SYNTHASE-INHIBITING HERBICIDES

(57) Abstract: Nucleotide sequences are disclosed that may be used to impart herbicide resistance to green plants. The sources of novel resistance were originally isolated in mutant rice plants. The sequences impart pre-emergence resistance, post-emergence resistance, or both pre-emergence resistance and post-emergence resistance to multiple herbicides. To date, resistance has been demonstrated against at least the following herbicides: imazethapyr, imazapic, imazapyr, imazamox, sulfometuron methyl, imazaquin, chlorimuron ethyl, metsulfuron methyl, rimsulfuron, thifensulfuron methyl, pyrithiobac sodium, tribenuron methyl, and nicosulfuron. Green plants transformed with these sequences are resistant to these herbicides and to derivatives of these herbicides, and to at least some of the other herbicides that normally inhibit acetohydroxyacid synthase (AHAS), particularly imidazolinone and sulfonylurea herbicides.

non-resistant individuals that escaped the foliar spray. Also important is the elimination of non-resistant individuals from late-sprouting seed. A plant that grows from a seed that sprouts after spraying will not be controlled by a herbicide having only foliar activity. Within two weeks, such a plant may reach a size that makes it appear to be a resistant mutant that survived the foliar treatment. If a second foliar spraying either is undesirable or is not feasible, an alternative is to leave a small area of the field unsprayed when applying the first application, to provide a direct standard for determining the size that resistant seedlings should achieve during the intervening period.

Using a herbicide with both soil and foliar activity also presents the opportunity to select efficiently for both pre-emergence and post-emergence resistance within the same individual plants. This selection is accomplished by applying sequential applications. If performed properly, the likelihood will then be high that individuals surviving sequential applications are resistant to both pre- and post-emergence treatments with that herbicide, rather than escapes.

As the selection procedure is in progress, care should be taken that the few surviving individuals are not eaten by birds or insects. Avoiding such predation is important for both post-emergence and pre-emergence treatments. Sound-making devices may be used to drive away birds, such as blackbirds, that consume rice seeds and small seedlings. Insects such as fall armyworms and rice water weevils also may kill small survivors, and the application of an insecticide on a preventative basis is frequently desirable. Daily monitoring of the situation should be undertaken if an investigator chooses not to use bird-discouraging devices or insecticides preventatively.

Assays for Total AHAS Activity

The procedures used to assay the activity of the acetohydroxyacid synthases from various rice lines as reported below were substantially as described in B.K. Singh *et al.*, "Assay of Acetohydroxyacid Synthase," *Analytical Biochemistry*, vol. 171, pp. 173-179 (1988), except as noted. In the first paragraph of Singh's "Materials and Methods," instead of corn suspension culture cells, shoot tissues from greenhouse-grown rice seedlings at the 3-4 leaf stage of development, or rice suspension culture cells were used. For shoot tissues, 40.0 or 50.0 grams (fresh weight) of tissue were extracted in the same manner for each of the breeding lines; for Cypress suspension cells, 16.0 grams of cells were used, harvested eight days after subculture. At the suggestion of the first author, B.K. Singh (personal communication), the desalting step mentioned at the bottom of Singh's first column under "Materials and Methods" was eliminated.

Pursuit™ herbicide (imazethapyr, also known as Newpath™) or Arsenal™ herbicide (imazapyr) was included in the "standard reaction mixture" for the AHAS assay in various concentrations. Colorimetric absorbance was measured at 520 nm. Checks were made of direct acetoin formation during the enzyme assay.

The following nine rice lines have been assayed in this manner to date: the non-resistant Cypress line (the parental line for some of the herbicide resistant lines), ATCC 97523, PTA-904, PTA-905, PTA-902, PTA-903, PTA-906, PTA-907, and PTA-908. Some assays were conducted at different times, and assays at some herbicide concentrations were repeated. Differences were noted among the lines with respect to total AHAS enzyme activity and the levels of herbicide resistance. In the modified Singh assay for total AHAS activity, using crude enzyme extract, in the absence of herbicide, most (but not all) of the herbicide-resistant lines expressed greater total AHAS activity than did the non-resistant Cypress line. Following treatment with the herbicides Pursuit™ (imazethapyr, also known as Newpath™) or Arsenal™ (imazapyr), the reduction in AHAS activity was greater in the Cypress line than in any of the resistant lines assayed. For the line that has appeared to have the highest resistance in testing to date, PWC23 (PTA-905), enzyme activity in the presence of very high herbicide levels (1000 µM of either imazethapyr or imazapyr) was similar to the enzyme activity of the nonresistant Cypress line in the absence of any herbicide. All the resistant lines assayed expressed resistance to both imazethapyr and imazapyr, while the nonresistant Cypress line was sensitive to both herbicides. Results are shown in Table 5. In Table 5, the first row ("No herbicide") is reported as absorbance at 520 nm. All other entries in a given column (i.e., for a given line of rice) are reported as a percentage of the absorbance for the same rice line in the absence of herbicide.

Table 5 -- Total AHAS Activity, Crude Enzyme Extracts, measured as absorbance at 520 nm

	Cypress	ATCC 97523 (93AS3510)	ATCC PTA-904 (PWC16)	ATCC PTA-905 (PWC23)	ATCC PTA-902 (CMC29)	ATCC PTA-903 (CMC31)	ATCC PTA-906 (WDC33)	ATCC PTA-907 (WDC37)	ATCC PTA-908 (WDC38)
No herbicide	0.766	0.837	0.713	1.107	0.811	1.038	0.851	1.226	0.822
50 µM imazethapyr	63%	101%	95%	99%	92%	84%	89%	92%	95%
100 µM imazethapyr (first replicate)	54%	92%	83%	88%	88%	82%	85%	86%	90%
100 µM imazethapyr (second replicate)	58%	91%	92%	93%	93%	87%	86%	---	---

1000 µM imazethapyr	56%	78%	78%	80%	84%	81%	82%	64%	66%
50 µM imazapyr	63%	91%	90%	95%	92%	86%	88%	84%	81%
100 µM imazapyr	57%	83%	83%	88%	84%	80%	74%	78%	78%
1000 µM imazapyr	45%	68%	76%	75%	75%	68%	73%	66%	66%

The results shown in Table 5 clearly show that each of the resistant lines listed in that Table (ATCC 97523, ATCC PTA-904, etc.) contains a resistant mutant AHAS enzyme. The lowest concentrations tested, 50 µM of imazethapyr or imazapyr, reduced the activity of the non-resistant line's AHAS to about 63% of control -- a reduction in activity that is more than ample to be lethal to plants in the field. By contrast, the resistant lines had AHAS activities ranging from 84% to 101% of control at these herbicide concentrations. Even at the highest herbicide concentrations tested, 1000 µM, herbicide activities in the resistant plants ranged from 64% to 84%, versus 45% or 56% for the non-resistant line. Put differently, each of the resistant plants showed higher AHAS activity at the extremely high herbicide concentration of 1000 µM than the AHAS activity of the non-resistant line at the lowest herbicide rate tested, 50 µM. In fact, the absolute activity exhibited by the most resistant line, PTA-905, at the highest 1000 µM herbicide rates tested (activities of 0.883 for imazethapyr and 0.834 for imazapyr) were higher than the AHAS activity for the non-resistant Cypress line in the absence of any herbicide (0.766).

The results given in Table 5 therefore clearly demonstrate that the herbicide resistance characteristics of at least the resistant rice lines listed in Table 1 were due to a resistant mutant AHAS enzyme.

Germination Inhibition Levels

Pre-emergence herbicide applications were tested to identify the levels of two different herbicides that would completely inhibit germination for several rice lines. Seed of each line tested was germinated in a plastic disposable petri dish containing 8 mL of herbicide solution and a layer of Whatman No. 4 filter paper. The fungicide Vitavax 200 at a concentration of 0.5 mL/L was added to the incubation solutions to inhibit fungal growth. Untreated controls were incubated in solutions containing fungicide but no herbicide. Twenty seeds were placed in each of 3 replicate dishes per treatment, and were incubated at 25°C under 16 hour : 8 hour light/dark photoperiods at a fluorescent light intensity of 15 micro-Einsteins per square meter per second. (One Einstein = 1 mole of photons.) Treatments were evaluated 11 days after incubation. The results of these pre-emergence experiments are shown in the Table 6, which indicates the